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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF

NOBUNAO IKEWAKI, ET AL.

: EXAMINER: LEWIS, P.

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: GROUP ART UNIT: 1623

FOR: BETA-1,3-1,6 GLUCAN  
(AUREOBASIDIUM MEDIUM)

DECLARATION UNDER 37 C.F.R. § 1.132

COMMISSIONER FOR PATENTS  
ALEXANDRIA, VIRGINIA 22313

SIR:

Now comes Nobunao Ikewaki who deposes and states:

- medical doctor course of
1. That I am a graduate of Tokai University and  
received a medical doctor degree in the year 1987  
Kitasato University, then Kyushu University
  2. That I have been employed by of Health and Welfare  
for 17 years as a Professor in the field of  
Clinical Immunology and Immunobiology.
  3. That the following experiments were carried out by me or under my direct  
supervision and control:
  4. *Aureobasidium*  $\beta$ -1,3-1,6-glucan (culture solutions/cultivated products) were  
produced by the method described in section 12 below. These products were produced from  
two different strains of *Aureobasidium*: control strain FERM-P4257 or by FERM-P18099  
(the strain of the present invention).  $\beta$ -1,3-1,6-glucan content in the compared culture  
solutions/cultivated products was standardized, so the effects of equivalent amounts of  $\beta$ -1,3-

1,6-glucan from each strain were compared in the data shown below. The different functional effects exhibited by  $\beta$ -1,3-1,6-glucan produced by these two different *Aureobasidium* strains provide strong evidence that  $\beta$ -1,3-1,6-glucans purified from these different *Aureobasidium* strains are structurally or compositionally distinct.

5. IL-8 Production Comparison. A comparison of the IL-8 produced by human peripheral blood mononuclear cells when stimulated with the  $\beta$ -1,3-1,6-glucan (cultivated product) obtained by FERM-P4257 (a control strain) or by FERM-P18099 (the strain of the present invention) was performed. The results of this comparison (below) show that the strain of the present invention FERM-P18099 induced greater amounts of IL-8 production than the control strain.

IL-8 production of human peripheral blood mononuclear cells by the cultivated product

Cultivation	IL-8 production power (pg/ml)	
	8 hours	24 hours
no addition	1263.2	10877.2
Addition of <i>Aureobasidium</i> culture solution	2052.6	22017.5
Addition of FERM-P18099	2821.3	28760.3

Influence of  $\beta$ -1,3-1,6 glucan (*Aureobasidium* culture solution) on IL-8 production in human peripheral blood mononuclear cells

Cultivation	Cytokine					
	IL-1 $\beta$ (pg/ml)	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)	INF- $\gamma$ (pg/ml)
no addition	< 10	< 0.8	< 2.0	< 0.2	12.6	< 0.1
addition of $\beta$ -1,3-1,6 glucan ( <i>Aureobasidium</i> culture solution)	< 10	< 0.8	< 2.0	0.4	100.7	< 0.1
addition of FERM-P18099	< 10	< 0.8	< 2.0	0.4	298.3	< 0.1

6. DNA Synthesis Comparison. A comparison of the amount of DNA synthesis induced in mouse spleen cells when stimulated with the  $\beta$ -1,3-1,6-glucan (cultivated product) obtained from FERM-P4257 (a control strain) or from FERM-P18099 (the strain of the present invention) was performed. The results are shown below:

Influence of FERM-P18099 cultivated product on DNA synthesis in mouse spleen cells  
DNA synthesis (cpm)

Cultivation	FERM-P4257 cultivated product	SI value	FERM-P18099 cultivated product	SI value
no addition	502.0		502.0	
addition	1044.3	2.0	1862.3	3.8

cpm: count per minute  
SI: stimulation index  
(addition group cpm + no addition group cpm)

Influence of  $\beta$ -1,3-1,6 glucan (*Aureobasidium* culture solution) on DNA synthesis power in mouse spleen cells

	DNA synthesis power				
	no addition	FERM-P4257	SI value	FERM-P18099	SI value
SA	624	851	1.4	2610	4.2
TS	444	2067	4.7	4430	10.6
YA	460	1232	2.7	2573	5.6
TA	272	4260	15.6	3964	14.6

cpm: count per minute  
SI: stimulation index  
(addition group cpm + no addition group cpm)

7. As shown above the  $\beta$ -1,3-1,6-glucan (culture solution) from strain FERM-P18099 of the present invention induced a significantly greater amount of DNA synthesis than the  $\beta$ -1,3-1,6-glucan (culture solution) from the control strain.

8. Cell Surface Antigen Comparison  $\beta$ -1,3-1,6-glucan (culture solution) obtained from FERM-P4257 (a control strain) or from FERM-P18099 (the strain of the present invention) exerted different effects on cell surface antigen expression in U937 cells (derived from a human histiocytic lymphoma). When these cells were cultured in the presence of the  $\beta$ -1,3-1,6-glucans from each strain, different levels of cell surface antigens were induced as shown below.

Influence of  $\beta$ -1,3-1,6 glucan (*Ascreobasidium* culture solution) on cell surface antigens of cultivated cell strain (U937)

strain	antibody isotype	receptor molecule	FERM-P4257 MFIs index (intensity index of fluorescence)	FERM-P18099
OKT-4	IgG2b	CD4	0.79	0.90
OKT-8	IgG2b	CD8	0.80	0.80
mNL-58A	IgG1	CD11a	1.61	1.71
SPV-L7	IgG1	CD11a	1.47	-
Bear-1	IgG1	CD11b	2.20	2.86*
Leu15	IgG2a	CD11b	2.28	2.99*
MY-4	IgG2b	CD14	0.87	0.91
AHN1.1	IgM	CD15	0.94	0.90
Leu11b	IgG	CD16	0.86	0.85
Huly-m13	IgM	CD17	0.94	0.90
IB4	IgG2a	CD18	1.12	1.06
H107	IgG2b	CD23	1.10	1.09
BA-1	IgM	CD24	0.92	0.92
IL-2R1	IgG2a	CD25	1.02	1.00
4B7R	IgG1	CD29	1.12	1.18
L133.1	IgG1	CD31	1.30	1.03
2H4	IgG1	CD45R	1.27	1.20
L25.3	IgG2b	CD49d	1.43	1.52
LB-2	IgG2b	CD54	2.38	2.91*
YH370	IgG	CD54	2.22	2.66*

B1/6	IgG1	CD106	1.05	1.05
Mike81	IgG2a	CD122	0.86	-
Sa-3	IgG3	HLA-class II	0.89	-
mN1-11	IgG1	ClqRp	2.07	2.78

9. These results clearly show the different effects produced by induction by the  $\beta$ -1,3-1,6-glucan (culture medium) produced by strain P-18099 of the present invention compared to the control *Aspergillus* strain.

10. Method for producing the  $\beta$ -1,3-1,6-glucans (culture solution/culture medium) of FERM-P4257 and FERM-P18099. The  $\beta$ -1,3-1,6-glucans (culture solutions/culture media) of FERM-P4257 and FERM-P18099 were produced by the following method. Each strain was respectively cultivated in a liquid medium containing vitamin E, pulverized ordinary rice bran 0.2 wt%, glucose 1.0 wt% and vitamin C 0.2 wt%. The pH of this medium was adjusted to 5.2. The inoculated medium was stirred and ventilated for 72 hours at 20°C. A supernatant was obtained from the cultivated medium by removing dead cells and insoluble solid matter. This supernatant was diluted with distilled water to adjust the  $\beta$ -1,3-1,6-glucan content of the diluted medium to 2.0 g/L. Thus, the final cultivated media were normalized so that the samples from both FERM-P4257 and FERM-P18099 contained the same amount of the  $\beta$ -1,3-1,6-glucan produced by each strain.

11. Standardization of the cultivated product or culture solutions obtained from FERM-P18099 and FERM-P4257 to 2.0 g/L. The cultivated products of the both strains (FERM-P4257 and FERM-P18099) used in the above comparisons were each measured for the quantity of  $\beta$ -1,3-1,6-glucan and then diluted with distilled water to a  $\beta$ -1,3-1,6-glucan concentration of 2.0 g/L according to the following method. The medium obtained from cultivation of each strain was diluted ten times by distilled water and a supernatant obtained

after removing insoluble matter by centrifugal separation at 40,000g for 30 minutes. The supernatant was mixed to a final concentration of 70% ethanol concentration to precipitate polysaccharide. The precipitate and soluble fractions were separated centrifugally. The precipitate obtained was re-dissolved in 20 mM citric acid-phosphoric acid buffer solution (pH6.0) to give a solution. This solution was added with 1/3 volume quantity of pullulanase suspension liquid (Wako company manufactured), which was previously diluted 1000 times by said buffer solution to undergo enzyme reaction at 30°C for 24 hours. The reacted product was used as the sample for measuring  $\beta$ -1,3-1,6-glucan. This sample was put into  $\alpha$ -cellulose membrane of fractional molecular weight 12,000 - 14,000 and subjected three times to dialysis against distilled water of 200 times quantity. Thereafter, the dialysis fluid within said membrane was added with ethanol until 80% of the final concentration to make polysaccharide precipitated, from which supernatant was removed by centrifugal separation. Total sugar quantity contained in the obtained precipitate was measured by phenol sulfuric acid method to determine the quantity of  $\beta$ -1,3-1,6-glucan.

#### 12. Taxonomic information about strain FERM-F4257

This fungus grows at 20-25°C and 25°C is the optimum cultivating temperature. 5°C-40°C of viable temperature and 5-7 of optimum viable pH at the time of cultivation using plate agar culture liquid culture, and shows dimorphism of hypha-yeast which is productive property likewise as strain of *Aureobasidium* genus according as its habitat. Further, the strain morphology can be divided into total three kinds of yeast type cell comprising a conidia which is a cell for proliferation and becomes an origin of hypha elongation, a yeast-like cell to which budding type conidia or segmentation type conidia has grown up and a chlamydo-spore having synthetic ability of melanin pigment which its produced by differentiation of hypha or yeast-like cell, and into total two kinds of hypha type cell

comprising a hypha in which cells are connected by thin septum and an expansion cell which has double septum and cell itself is thickened. ....

In this fungus, hypha elongation and budding from blastospore actively take place in a habitat abundant in nutrition, vacuole may be recognized inside of the cell with the lapse of time, elongated hypha has double septum and the cell itself is also shifted to a state of thickened expansion cell. On the other hand, when cultivated in a state of poor nutrition, segmentation of expanded hypha comes to be observed and at the time of deterioration of other environmental factors or further poorer nutrition state, chlamydo-spore, which has double cell wall further larger than expanded cell and is excellent in biophylaxis, comes to be recognized.

In case this fungus is cultivated on potato dextrose (Nissui Seiyaku Manufactured) - plate-agar culture medium at 25°C-30°C for two weeks, colony of 5-6 cm in diameter, in which glass by viscous matter coating surface and standing crops by hypha elongations are observed, are formed, the colony overall is black. From these characteristics, it can be classified as an *Aureobasidium* sp. (Taxonomic information for *Aureobasidium* strain P-18099 is provided in the specification on pages 8-10.)



13. The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing therefrom.

14. Further deponent saith not.

Nobunao Ikewaki  
Signature

June 13, 2004  
Date

CAUTION: 21505105-002.DOC